C5

catabolism without increasing incidence of intracerebral hemorrhage in the human subject.--

A mark up copy of the claims is attached hereto as **Exhibit 1**.

REMARKS

Claims 1-13 and 16-26 are currently pending in the above-identified application. Claims 1, 3 and 9-13 are in condition for allowance. By this Amendment applicants have hereinabove canceled claims 21, 25 and 26, amended claims 2, 4, 8 and 17, and added new claim 27. Consequently, after entry of this Amendment claims 1-13, 16-20, 22-24 and 27 will be pending with claims 1,3 and 9-13 in condition for allowance. Applicants maintain that the amendments to the claims raise no issue of new matter. Support for amended claim 2 can be found in the specification as originally filed at, inter alia, page 6, lines 10-12. Support for amended claim 4 can be found in the specification as originally filed at, inter alia, page 12, lines 11-14 and page 21, lines 16-20. Support for amended claim 8 can be found in the specification as originally filed at, inter alia, page 15, line 35 to page 16, line 2. Support for amended claim 17 can be found in the specification as originally filed at, inter alia, page 14, lines 19 to 32. Support for new claim 27 can be found in the specification as originally filed at, inter alia, page 6, lines 2-12. Accordingly, applicant respectfully requests that these amendments be entered.

In the January 30, 2002 Office Action the Examiner stated that claim 17 is objected because the claim recites "and/or" in line 15 section (d), and that changing "and/or" to "or" and adding "or

both" after the phrase "fibrin deposition" in line 15 would be remedial.

In response, applicants have amended claim 17 as suggested by the Examiner.

Claim Rejections Under 35 U.S.C. §112 (Second Paragraph)

The Examiner stated that claims 2, 4, 8 and 16 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. The Examiner stated that the term "mutated" in claim 2 is vague and renders the claim indefinite and that it is unclear as to the metes and bound of what would be considered "mutated"? The Examiner stated that if the term "mutated" means substitution, addition, or deletion of amino acids within SEQ ID NO:1, then claim 2 fails to provide further limitation from claim 1. The Examiner also stated that claim 1 encompasses a polypeptide that comprises an active polypeptide fragment of SEQ ID NO:1 and therefore does not encompass substitution, deletion other than truncation, or addition of amino acids within SEQ ID NO:1.

In response, without conceding the correctness of the Examiner's position, applicants have amended claim 2 to remove the term "mutated". In addition, applicants have added new independent claim 27 directed to mutated forms of CD39.

The Examiner further stated that the phrase "having IL-2 as its leader sequence" in claim 4 is vague and renders the claim

indefinite and that IL-2 is a cytokine that has a leader sequence. The Examiner stated that it is unclear how one would use the whole IL-2 as a leader sequence, and that claim 5 depends on claim 4 but fails to clarify the indefiniteness.

In response, applicants have hereinabove amended claim 4 to refer to the "leader sequence of IL-2".

The Examiner stated that the phrase "the CD39 polypeptide or its fragment is linked to a pharmaceutical acceptable carrier" in claim 8 is vague and renders the claim indefinite. The Examiner stated that it is unclear how the CD39 polypeptide or its fragment is going to link to a pharmaceutically acceptable carrier, and that the specification fails to define the linking of a CD39 polypeptides or its fragment to a pharmaceutically acceptable carrier. The Examiner stated that claim 16 depends on claim 8 but fails to clarify the indefiniteness.

In response, without conceding the correctness of the Examiner's position, applicants have hereinabove amended claim 8, including removing the term "linked".

The Examiner stated that claims 17-26 are rejected under 35 U.S.C. §112, second paragraph, as being incomplete for omitting essential steps, such omission amounting to a gap between the steps, and that the omitted steps are: step (e) of claim 17 fails to specifically point out the condition that would indicate the compound is capable of treating or preventing thrombotic or ischemic disorders in a subject, such as a decrease of platelet deposition would indicate the compound can inhibit platelet aggregation and is capable of

treating or preventing thrombotic or ischemic disorders.

In response, without conceding the correctness of the Examiner's position, applicants have hereinabove amended claim 17 to specify the condition indicating that the compound is capable of treating or preventing thrombotic or ischemic disorders in a subject.

Claim Rejections Under 35 U.S.C. §112 (First Paragraph)

The Examiner stated that the claims 2,6,7,21 and 25 are rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Examiner stated that claims 2, 6 and 7 read on using mutated form of CD39 polypeptide, or a polypeptide comprising amino acid position 1-50 of SEQ ID NO:2 or comprising 20-80 amino acid residues of SEQ ID NO:1 that mimic the active site, to treat or prevent stroke in a human subject susceptible to intracranial hemorrhaging. The Examiner stated that the specification discloses that SEQ ID NO:1 is the coding sequence of CD39 and SEQ ID NO:2 is a variant of CD39. The Examiner stated that the specification provides general disclosure on the composition of other variants, such as that they have substitutions, deletions, or insertions which do not abolish the biological activity associated with CD39 and which may have increased potency, bioavailability, stability or decreased toxicity. The Examiner also stated that in the various exemplifications provided in the specification, only the

polypeptide of SEQ ID NO:2 is utilized (soluble CD39 lacking C-terminal and N-Terminal transmembrane domains).

The Examiner further stated that the scope of the claims encompasses a genus of structural variants of SEQ ID NO:1, and the genus is highly variant because a significant number of structural differences between genus members is permitted. The Examiner stated that a polypeptide comprising a mutated form of SEQ ID NO:1 via substitution, deletion or addition, or an active fragment comprising amino acid position 1-50 of SEQ ID NO:2 or comprising 20-80 amino acid residues of SEQ ID NO:1 encompasses numerous unknown and unidentified polypeptide sequences that differ dramatically from the polypeptide sequence of SEQ ID NO:1. The Examiner stated that the structural features that could distinguish CD39 fragments, having activity in inhibiting adenosine diphosphate (ADP)-mediated platelet aggregation by increasing ADP catabolism, in the genus from others in the polypeptide class are missing from the disclosure and that the specification fails to disclose the active site of CD39 that contribute to its activity in inhibiting ADP-mediated platelet aggregation by increasing ADP catabolism. The Examiner also stated that no common structural attributes identify the members of the genus and that since the disclosure fails to describe common attributes or characteristics that identify members of the genus, and because the genus is highly variant, the disclosure of SEQ ID NO:1 and 2 is insufficient to describe the genus. The Examiner stated that this limited information is not sufficient to reasonably convey to one skilled in the art that applicants were in possession of the numerous variants of SEQ ID NO:1 as claimed in the present invention, and concluded that the written description requirement is not satisfied for the genus.

In response, applicants maintain that the structural features that can distinguish CD39 polypeptide active fragments are disclosed in the application. The specification refers to the deletion of apyrase conserved regions (ACRs) to remove CD-39 activity (see specification page 18, line 33 to page 19, line 1, and at page 27 line 35 to page 29 line 5. See also reference #13, Handa et al., 1996, attached hereto as **Exhibit 2**) and that fragments with four ACRs retain activity (see page 36, lines 18-23). All these ACRs are found in SEQ ID NO:1 and SEQ ID NO:2 in the sequence listing and on page 9 of the specification, second and third paragraphs. Furthermore, it was known in the art at the time of filing that deletions in ACR 1 or 4 reduce biological activity, e.g. Schulte et al. February, 1999 showed that ACR 1 or 4 are needed for activity. Gayle (Exhibit G of November 22, 2000 Information Disclosure Statement and reference #11 in the specification, attached hereto as **Exhibit 3**) states that the four ACRs are characteristic of the members of the apyrase family and accepts their role in ecto-ADPase activity (see Gayle, page 1857). The ACRs are required for activity, and are therefore a necessary feature in any variants. Applicants maintain that the specification's disclosure of both functional and structural characteristics is sufficient to reasonably convey to one skilled in the art that applicants were in possession of the variants of SEQ ID NO:1 as claimed in the present invention, and that the written description requirement is satisfied for the genus claimed.

The Examiner stated that claims 21 and 25 are directed to a compound identified by the method of claim 17, and a pharmaceutical composition comprising said compound and a pharmaceutically acceptable carrier and that the claims encompass a genus of organic

compounds that could be identified via the method of claim 17, which reads on far more than compounds structurally and functionally related to CD39.

The Examiner stated that the specification only discloses that SEQ ID NO:1 is the coding sequence of CD39, and SEQ ID NO:2 is a variant of CD39 (specification, page 9, lines 3-5). The Examiner stated that organic compounds and polypeptides, such as CD39, have different physical and chemical properties and they also differ in their functional characteristics. The Examiner stated that the specification fails to disclose the physical and chemical properties of the claimed compound and what functional characteristics of an organic compound would inhibit platelet aggregation or leucocyte accumulation by increasing ADP catabolism and not increase incidence of intracerebral hemorrhage. The Examiner also stated that the structural feature of a compound that would inhibit platelet aggregation or leucocyte accumulation by increasing ADP catabolism was not disclosed, and that one skilled in the art at the time of the invention would not be able to distinguish the compound having the ability in inhibiting platelet aggregation from the compound that does not have such ability based solely on the description in the specification of such compound, i.e. the specification discloses no structural features of such compound. The Examiner stated that the specification fails to provide sufficient description of the claimed compounds that could be identified by claim 17. The Examiner further stated that the limited information of SEQ ID NOs:1 and 2 is not sufficient to reasonably convey to one skilled in the art that applicants were in possession of the genus of compounds or compositions comprising said compounds as claimed in the present invention, and concluded

that the written description requirement is not satisfied for the genus.

In response, without conceding the correctness of the Examiner's position, applicants have amended canceled claims 21 and 25.

Claim Rejections Under 35 U.S.C. §112 - (First Paragraph)

The Examiner stated that claims 2,6 and 7 are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for the use of soluble CD39 in the treatment and prevention of thrombotic and ischemic disorders in mice and BIBU52 in rhesus and marmoset monkeys (Guth et al., abstract), does not reasonably provide enablement for the use of any mutated CD39 fragment, an active fragment comprising amino acid position 1 to 50 of SEQ ID NO:2 or an active fragment comprising about 20-80 amino acid residues of SEQ ID NO:1 in treating or preventing stroke. The Examiner stated that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

The Examiner stated that claims 2, 6 and 7 read on using a mutated form of CD39 polypeptide, or a polypeptide comprising amino acid residues 1-50 of SEQ ID NO:2 or comprising 20-80 amino acid residues of SEQ ID NO:1, to treat or prevent stroke in a human subject susceptible to intracranial hemorrhaging. The Examiner stated that the specification fails to disclose the identity of any CD39 variant, other than the exemplified SEQ ID NO:2, with the ability to inhibit platelet aggregation by increasing ADP

catabolism. The Examiner stated that although the specification provides a general disclosure on the methods of generating additional CD39 variants (specification, p. 8, line 25-p. 12, line 10), said disclosure does not provide specific details on the structural feature of a fragment which is necessary to inhibit platelet aggregation. The Examiner stated that, further, the amino acid sequence of a protein determines its structural and functional properties, and predictability of which amino acids can be removed from a protein's sequence and still result in similar activity is extremely complex, and well outside the realm of routine experimentation, because accurate predictions of a protein's structure from mere sequence data are limited. Rudinger, 1976 (Peptide Hormones, Edited by Parsons, University Park Press, Baltimore, p. 1-7), points out that "The significance of particular amino acids and sequences for different aspects of biological activity cannot be predicted a priori but must be determined from case to case by painstaking experimental study" (e.g. p6), and Kaye et al., 1990 (Proc. Natl. Acad. Sci. USA, Vol. 87, pp. 6922-6926) teaches that "A single amino acid substitution results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding" (e.g. Title). The Examiner stated that one skilled in the art would not know how to make and use the claimed CD39 variants or polypeptide comprising amino acid position 1-50 of SEQ ID NO:2 or comprising 20-80 amino acid residues of SEQ ID NO:1 that have ability to inhibit platelet aggregation by increasing ADP catabolism.

The Examiner stated that therefore, in the absence of teachings disclosing the ability of any variant other than the soluble form of CD39 (SEQ ID NO:2) to inhibit platelet aggregation or ADPase

activity in vivo, or even to maintain its biological activity in vivo for long periods of time following i.v. administration (see Gayle et al., p. 183, column 1), and the unpredictability of biological function of a polypeptide from mere amino acid sequence, one skilled in the art at the time of the invention would have to engage in undue experimentation to practice over the full scope of the invention claimed.

In response, applicants note that the sequence modifications stated in the specification are encompassed within those limits referred to by the Examiner. The specification states (e.g. see page 8 line 33 - page 9 line 3) that substitutions that would typically have minimal influence on secondary structure and hydrophobicity of the sol CD39 would be made, i.e. conservative substitutions. Such substitutions are predictable within the limits of current knowledge. This is exemplified by Schulte et al, 1999 (**Exhibit 3**) which demonstrates modifying the CD39 sequence by the addition of an eight residue FLAG tag to a CD39 which does not change CD39 ADPase activity nor its antigenic structure. In addition, applicants note that there is no recitation in the claims that the variants must have similar activity to the exemplified sequences, only that the variants have some activity.

Applicants further note that the citation used by Examiner to support the contention that the effect amino acid substitutions cannot be predicted before hand but must be determined "by painstaking experimental study" was written more than 20 years before the filing date of this application and does not reflect the state of the field at the time of filing.

Furthermore, applicants note that only CD-39 variants active in platelet aggregation will be used, as determined by platelet aggregation assay. One of ordinary skill in the art would have routinely carried out platelet aggregation assay (as described in the specification at p23, 132) and/or in vivo assay to assess the ability of a candidate CD39 variant without undue experimentation (such assays are so routine that automated aggregometers are manufactured commercially), and thus identify those variants which were active. Such simple and routine and automated experimentation would not be undue.

Moreover, it is well settled law that enablement is not precluded by the necessity for some experimentation. In re Wands, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). The key word in this regard is "undue" not "experimentation." Id., citing in re Angstadt, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976). Thus

> [t]he determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the claimed invention and the state of the art. [citations omitted] The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

Wands at 1404, citing in re Jackson, 217 U.S.P.Q. 804, 807 (B.P.A.I. 1982).

Finally, applicants note that the claims do not recite a requirement for the variant to "maintain its biological activity in vivo for long periods of time following i.v. administration" as suggested by the Examiner.

The Examiner stated that claims 17-26 are rejected under 35 U.S.C.

§112, first paragraph, because the specification, while being enabling for a transgenic mouse comprising a homozygous deletion in CD39 and its use in identifying compounds which inhibit platelet aggregation via the ADP pathway, does not reasonably provide enablement for the use of an animal model in testing for compounds which inhibit platelet aggregation via any pathway other than ADP catabolism pathway. The Examiner stated that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

The Examiner stated that claims 17-26 are directed to a method for determining whether a compound inhibits platelet aggregation or leukocyte accumulation by increasing ADP catabolism and does not increase incidence of intracerebral hemorrhage by inducing thrombotic or ischemic disorders in an animal, administering a test compound to said animal and measuring platelet deposition and/or fibrin deposition in ischemic tissue in the animal, the compound identified by said method, and a composition containing said compound. The Examiner further stated that the specification fails to provide an enabling disclosure for the use of an animal model to test for compounds which inhibit platelet aggregation via pathways other than ADP catabolism by measuring said compound effect on all types of platelet deposition. Platelet aggregation during thrombosis is induced by collagen, ADP, and a thrombin receptor-activating peptide (Guth et al., abstract). The Examiner stated that the soluble CD39 has been disclosed by the art to be an ADPase and that the claimed invention is not enabled for testing compounds which are not previously known to be ADPase's because one skilled in the art at the time of the invention would not be able to discern if any platelet aggregation resulting from use of a test compound acted via collagen or thrombin receptor-activating peptide pathway or ADPase pathway. The Examiner stated that the

specification fails to teach the manner of blocking the collagen or thrombin receptor-activating pathways in the animal model to ensure that any effect the compound had on the inhibition of platelet aggregation was through the ADPase catabolism pathway as regarded by the claims and that the specification also fails to disclose assays to test specifically for inhibition of platelet aggregation via the ADPase pathway versus via the inhibition of the collagen or thrombin receptor-activating pathways. The Examiner stated that, therefore, one of skilled in the art at the time of the invention would be required to engage in undue experimentation to identify the pathway by which any compound might inhibit platelet aggregation in the claimed animal model or to amend the method to add steps that would discriminate between compounds acting on ADP catabolism and those acting on other pathways that inhibit platelet and fibrin depositions.

In response, without conceding the correctness of the Examiner's position, applicants have amended claim 17. Applicants note that amended claim 17 is directed to a method for determining if compounds with ADP catabolic activity can treat ischemic or thrombotic disorder without causing an increase in intracerebral hemorrhage.

Claim Rejections Under 35 U.S.C. §102

The Examiner stated that claims 21, 25 and 26 are rejected under 35 U.S.C. §102(b) as being clearly anticipated by Gayle et al., 1998 (The Journal Clinical Investigation, Vol. 10, No. 9, p. 1851-1859). The Examiner stated claims 21, 25 and 26 are directed to a compound identified by the method of claim 17, a pharmaceutical composition comprising said compound, and a pharmaceutical composition comprising a CD39 polypeptide or an active fragment thereof and a pharmaceutical acceptable carrier.

The Examiner stated that Gayle discloses preparation of a recombinant soluble form of CD39 by affinity purification and demonstrate its antithrombotic activity in vitro by catabolizing ADP and resulting in the inhibition of platelet aggregation, and that it remained biologically active in vivo while circulating for prolonged periods of time (e.g. abstract, Figure 1, p. 1852, 1858). The Examiner stated that claim 21, 25 and 26 are composition claims and that the method by which the composition is obtained does not carry weight in 35 U.S.C. §102 rejection. The Examiner further stated that the soluble form of CD39 can inhibit platelet aggregation and that the solution containing the soluble form of CD39 is a pharmaceutical acceptable carrier, and thus, claims 21, 25 and 26 are clearly anticipated by Gayle.

The Examiner stated that The Examiner stated that claims 21 and 25 are rejected under 35 U.S.C. §102(b) as being anticipated by Guth et al., 1997 (Journal of Cardiovascular Pharmacology, Vol. 30, p.261-272). The Examiner stated that claims 21 and 25 are directed to a compound identified by the method of claim 17, and a pharmaceutical composition comprising said compound and a pharmaceutical acceptable carrier.

The Examiner stated that Guth discloses a nonpeptide molecule, BIBU52, that can inhibit the aggregation of human platelets in platelet-rich plasma induced by collagen, ADP, and a thrombin-receptor activating peptide. BIBU52 inhibits aggregation in plasma from rhesus and marmoset monkeys but not in rat plasma (e.g. abstract). The Examiner also stated that the method by which the composition is obtained does not carry weight in 35 U.S.C. §102 rejection. The BIBU52 compound can inhibit platelet aggregation and the solution containing the BIBU52 compound is a pharmaceutical acceptable carrier. The Examiner stated that thus, claims 21 and 25 are clearly anticipated by Guth.

In response, without conceding the correctness of the Examiner's position, applicants have canceled claims 21, 25 and 26.

Claim Rejections Under 35 U.S.C. §103

The Examiner stated that claims 17 and 20-24 remain rejected under 35 U.S.C. §103(a) as being unpatentable over Guth et al., 1997 (Journal of Cardiovascular Pharmacology, Vol. 30, p.261-272) in view of Gayle et al., 1998 (The Journal Clinical Investigation, Vol. 10, No. 9, p. 1851-1859) and Choudhri et al., 1998 (J. Clin. Invest. Vol. 102, No. 7, p. 1301-1310, IDS-exhibit 8).

The Examiner stated that Guth discloses the use of three different animal models of recurrent arterial thrombus formation to test the efficacy of a compound, e.g. BIBU52 to inhibit ADP driven platelet aggregation in rhesus and marmoset monkeys. The Examiner stated that Guth does not disclose the use of CD39 or that the compound does not increase the incidence of intracerebral hemorrhage.

The Examiner stated that Gayle discloses a recombinant soluble form of CD39 and demonstrate its antithrombotic activity in vitro by catabolizing ADP and resulting in the inhibition of platelet aggregation, and that it remained biologically active *in vivo* while circulating for prolonged periods of time (e.g. abstract, Figure 1, p. 1858).

The Examiner stated that Choudhri discloses testing the effects of a potent antiplatelet agent given both before and after the onset of middle cerebral arterial (MCA) occlusion in a murine model of stroke and shows a novel inhibitor of the glycoprotein IIb/IIIa receptor (SDZ GPI 562) exhibits a dose-dependent reduction of cerebral infarct volumes as well as improvement in postishchemic cerebral blood flow. The Examiner stated that Choudhri also teaches

GPI 562 causes a dose-dependent increase in tail vein bleeding time, but intracerebral hemorrhage (ICH) is not significantly increased at therapeutic doses (e.g. abstract).

The Examiner stated that it would have been obvious for one of ordinary skill in the art at the time of the invention to utilize an animal model of thrombosis to test for the effect of a potential therapeutic compound, such as soluble form of CD39 or GPI 562, on inhibiting ADP driven platelet aggregation and without increasing incidence of intracerebral hemorrhage, and that it would have been obvious for one of ordinary skill to compare the results of an animal model with and without the treatment of a test compound in order to determine the effects of said test compound.

The Examiner stated that one of ordinary skill at the time the invention was made would have been motivated to use the soluble CD39 as the test compound in the model set forth above because Gayle teaches it inhibits platelet aggregation *in vitro* by catabolizing ADP and that it remains biologically active *in vivo*, and Choudhri teaches antiplatelet agent, such as GPI 562, may cause increasing bleeding time in tail vein but ICH is not significantly increased at therapeutic doses, thus displaying the potential to inhibit platelet aggregation in an animal under thrombotic conditions and without increasing the incidence of ICH.

In response, applicants respectfully traverse Examiner's rejection and maintain that claims 17 and 20-24 are patentable over Guth et al. (1997) in view of Gayle et al. (1998) and Choudhri et al., 1998. Applicants note that according to M.P.E.P. §2143.01 there must be motivation to combine references (see In re Rouffet, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-1458 (Fed. Cir. 1998). Applicants further note that the Guth paper neither mentions or alludes to ICH. The requirement that the drug not increase ICH is

an element of claim 17 and its dependents. Animal thrombus models that do not measure ICH are not relevant as such models do not test for what is a necessary element of the claims.

Further, Guth at el. only teaches BIBU52, a compound which works via IIb/IIIa antagonism. In contrast, claim 17 and dependents require that the drug increase ADP catabolism. There is no teaching that BIBU52 increases ADP catabolism. In fact, to applicant's knowledge the phenomenon of a IIb/IIIa antagonist also increasing ADP catabolism has never been reported. Gayle et al. teaches that solcd39 has an "independent mode of action" (from No IIb/IIIa antagonists), i.e. increasing ADP catabolism (as is required by claim 17 and dependents). Thus there is no motivation to combine the teachings of Gayle et al. with respect to ADPases with the teachings of the animal models in Guth et al. which apply to Group IIb/IIIa antagonists, as the they are two different classes of compounds. These two classes of compounds have two different actions and such independent modes of action (i) teach away from combining the references and (ii) do not support a reasonable expectation of success in combining the teachings.

Applicant further notes that other members of the IIb/IIIa class have been shown to increase ICH, in fact small excesses of IIb/IIIa agonists can give serious ICH as stated in the introduction of the specification at page 2 lines 8-15.

Choudri teaches a group IIb/IIIa antagonist compound that has no shown ability to "increase ADP catabolism". Increasing ADP catabolism is a required element of claim 17 and dependents. As stated hereinabove, there is no evidence cited by Examiner (nor is applicant aware of any such evidence in existence) that IIb/IIIa antagonists can increase ADP catabolism. In contrast Gayle et al. teaches a compound whose mechanism of action is to increase ADP

catabolism. There is no motivation to combine the teaching of a compound which increases ADP catabolism (Gayle) with the teaching of a member of a class of compounds not taught to increase ADP catabolism (GSZ 251, Choudri) in order to conclude that the former does not increase ICH just because the latter does not increase ICH. Further, there is no reasonable expectation of success.

In summary, applicants note there is no motivation to combine the cited references, and no reasonable degree of success expected as the teachings with respect to one class of compound are not readily transferable to a class of compound with a different mode of action.

The Examiner stated that claims 25 and 26 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Guth et al., 1997 (Journal of Cardiovascular Pharmacology, Vol. 30, p. 21-272) in view of Gayle et al., 1998 (The Journal Clinical Investigation, Vol. 10, No. 9, p. 1851-1859) and Choudhri et al., 1998 (J. Clin. Invest. Vol. 102, No. 7, p. 1301-1310) as applied to claims 17 and 20-24 above and further in view of Beaudoin et al. (US Patent No. 5,798,241).

The Examiner stated that the teachings of Guth, Gayle and Choudhri are as discused above, and that Beaudoin teaches the use of a composition comprising mammalian ATP diphosphohydrolase with a pharmaceutically acceptable carrier to reduce platelet aggregation and thrombogenicity (claim 5, col. 9, lines 34-37).

The Examiner stated that it would have been obvious for one of ordinary skill in the art at the time of the invention to utilize a compound identified from an animal model of thrombosis which displays the activity of catabolizing ADP and without increasing incidence of intracerebral hemorrhage according to the collective

teaching of Guth, Gayle and Choudhri, in a pharmaceutical composition as taught by Beaudoin in order to inhibit platelet aggregation in an animal under thrombotic conditions and without increasing the incidence of ICH.

In response, applicants have noted hereinabove why there is no motivation to combine the teachings of Gayle et al., Guth et al. and Choudri et al., and that there is no reasonable expectation of success in combining the teachings with that taught by Beaudoin et al.

Applicants gratefully acknowledge Examiner's statement that claims 1,3 and 9-13 are in condition for allowance and respectfully request that the Examiner withdraw the objections to the remaining claims in light of the arguments presented hereinabove.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the enclosed total fee of $469.00, including a $460 fee for a three-month extension of time and $9.00 fee for extra claims presented, is deemed necessary in connection with the filing of this Amendment. If any other fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

John P. White

John P. White
Registration No. 28,678
Attorney for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

John P. White 7/23/02

John P. White Date
Reg. No. 28,678

Mark Up copy of Amendments to the Claims

Claim 2 has been amended as follows:

--2. (2x Amended) The method of claim 1, wherein the active polypeptide fragment of CD39 polypeptide is administered and is a [mutated or a] truncated form of the CD39 polypeptide.--

Claim 4 has been amended as follows:

--4. (Amended) The method of claim 3, wherein the CD39 polypeptide is a recombinant CD39 polypeptide having <u>an</u> IL-2 [as its] leader sequence.--

Claim 8 has been amended as follows:

--8. (Amended) The method of claim 1, wherein the CD39 polypeptide or its fragment is [linked to] <u>administered as a composition comprising the CD39 polypeptide or its fragment and</u> a pharmaceutically acceptable carrier.--

Claim 17 has been amended as follows:

--17. (3x Amended) A method for determining whether a compound <u>which increases ADP catabolism</u> inhibits platelet aggregation or leukocyte accumulation [by increasing ADP catabolism] and does not increase incidence of intracerebral hemorrhage, so as to treat or prevent thrombotic or ischemic disorder in a subject, comprising:

(a) administering the compound to an animal, which is a model for the thrombotic or ischemic disorder, before,

concurrently with or after step (b);

(b) inducing the thrombotic or ischemic disorder in the animal;

(c) measuring the stroke outcome and the incidence of intracerebral hemorrhage in the animal;

(d) measuring platelet [and/]or fibrin deposition or both in ischemic tissue in the animal; and

(e) comparing the stroke outcome and incidence of intracerebral hemorrhage and the platelet [and/]or fibrin deposition or both in the presence of the compound with in the absence of the compound [so as to determine whether] wherein a decrease in platelet or fibrin deposition or both and no increase in intracerebral hemorrhage indicates that the compound is capable of treating or preventing the thrombotic or ischemic disorder in the subject.--

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 218, 916–923 (1996)
ARTICLE NO. 0162

Purification and Cloning of a Soluble ATP-Diphosphohydrolase (Apyrase) from Potato Tubers (*Solanum tuberosum*)[1]

Masahisa Handa[2,3] and Guido Guidotti

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, 02138

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A soluble ATP-diphosphohydrolase (apyrase, EC 3.6.1.5) has been purified from potato tubers, *Solanum tuberosum*, to a specific activity of 10,000 μmol P_i/mg/min. The cDNA corresponding to the potato apyrase has been isolated and termed RROP1. The deduced amino acid sequence contains a putative signal sequence, two hydrophobic regions at the carboxy terminus, two potential Asn-linked glycosylation sites, and four regions in the amino-terminal half that we term ACR (apyrase conserved regions) 1–4 that are highly conserved in known apyrases and related enzymes: garden pea nucleoside triphosphatase, *Toxoplasma gondii* nucleoside triphosphate hydrolases, and *Saccharomyces cerevisiae* golgi guanosine diphosphatase. A yeast 71.9-kDa hypothetical protein on chromosome V, a *Caenorhabditis elegans* hypothetical 61.3-kDa protein on chromosome III, and human CD39, a lymphoid cell activation antigen, also share the conserved ACR regions, but their ability to hydrolyze nucleotides has not been assessed. © 1996 Academic Press, Inc.

ATP-diphosphohydrolase (apyrase) catalyzes the hydrolysis of phosphoanhydride bonds of nucleoside tri- and di- phosphates in the presence of divalent cations. This enzyme is distinct from other phosphohydrolases in that it has a high specific activity, broad nucleotide substrate specificity, broad divalent cation requirement, and insensitivity to known inhibitors of various ATPases (P-type, F-type, and V-type), phosphatases, and adenylate kinase. The family of proteins that share these characteristics has been termed E-type ATPases (1). Apyrase activities are found in a wide variety of organisms, tissue types, and cell types, as reviewed by Plesner (1). In animals, the enzyme exists as a plasma membrane ecto-ATPase in cell types such as rat hepatocytes (2), rabbit skeletal muscle transverse tubules (3), and chicken gizzard smooth muscle (4). Recently, soluble apyrases from the dense granules of a protozoan parasite, *Toxoplasma gondii* (5,6), and from mosquito saliva (*Aedes aegypti*) (7) have been cloned.

The physiological function(s) of the apyrases are not known, but various investigators have put forth hypotheses based on the extracellular nature of the enzymatic activity and the physiology of extracellular nucleotides. Possible functions include: 1) the concomitant regulation of P_2-purinergic receptors that recognize ATP and P_1-purinergic receptors that recognize adenosine; 2) the salvaging of extracellular adenosine through the sequential hydrolysis of ATP to AMP by apyrase, AMP to adenosine by 5′-nucleotidase, and subsequent import through nucleoside transporters; 3) the regulation of ecto-kinase substrate concentration, and 4) the inhibition of ADP-induced platelet aggregation (1,2,8–12).

Potato apyrase was first characterized by Kalckar (13) as an adenylpyrophosphatase and was later termed apyrase by Meyerhof (14). A number of isoapyrases with varying kinetic and subcellular localization properties have been identified from different varieties of *Solanum tuberosum* (15–19). Potato apyrase has been speculated to be a regulator of various steps involved in starch synthesis, as glycosyltransferases, ADP-glucose pyrophosphorylase, and other starch metabolism enzymes are regulated by the levels of either ATP, ADP, or P_i (20–22).

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[2] Corresponding author. Fax: (617) 495-8308. E-mail: mhanda@fas.harvard.edu.
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We have cloned the cDNA for the potato apyrase to further investigate the structure/function relationship of the potato apyrase polypeptide. Sequence comparisons with other apyrases and related proteins show the existence of several conserved regions, suggesting the apyrases are an evolutionarily related family of proteins.

MATERIALS AND METHODS

Apyrase purification. The soluble apyrase was isolated from red skin potatoes (*Solanum tuberosum*) obtained from a local food market (Star Market, MA). The enzyme was purified according to Kettlun *et al.* (21) with an additional sucrose density centrifugation step. The sucrose gradient consisted of a 10%–30% continuous gradient in 50 mM potassium succinate, 400 mM potassium chloride, 1 mM thioglycolic acid, pH 4.0. The gradient was centrifuged at 180,000 x g in an SW 50.1 rotor for 24 hours.

Apyrase activity. Apyrase activity was determined by the release of orthophosphate from ATP as described by Fiske and Subbarow (23). The enzymatic activity is expressed as μmol P_i/mg of protein/minute. Approximately 10 ng – 20 μg of total protein, quantitated using the method described by Bradford (24), from the various purification steps were used in the assay.

Microsequencing. The purified apyrase was analyzed for amino terminal and trypsin digested peptide sequences by William S. Lane (Harvard Microsequencing Facility, Harvard University, Cambridge, MA). The tryptic digested peptides were separated on an HPLC column and five peptides were sequenced.

PCR amplification. Degenerate oligonucleotides were designed from the amino and carboxy terminal ends in the sense and antisense orientations, respectively, of the two longest peptides (Fig. 2, peptides #1 and #2).

PCR reactions were performed as described by Maniatis *et al.* (25) at an annealing temperature of 50°C using single stranded potato tuber cDNA as template. Total RNA was isolated from potato tubers (var. Red dale, Johnny's, Maine) using the method of Logemann *et al.* (26). Poly-A RNA was further purified using the PolyATract mRNA Systems II Kit (Promega). Single stranded cDNA was synthesized from ~10 μg poly-A RNA using Superscript Reverse Transcriptase (Gibco BRL).

cDNA sequences corresponding to both peptides were amplified, cloned into the polylinker site of pGEM 3Zf(–), and sequenced from the T7 and Sp6 promoters using the Sequenase Version 2.0 Kit (U.S. Biochemical).

Non-degenerate sense and antisense primers from peptides #1 and #2, respectively, produced a PCR amplified product of 131 bp. This DNA was sequenced as above. All oligonucleotides were made by Michael Berne (Tufts Microchemistry Facility, Tufts University Medical School, Boston, MA).

cDNA library screening. Potato tuber cDNA libraries in λ_{ZAP} were generously provided by Dr. Thomas Okita (Washington State University, WA) and Dr. Normand Brisson (University of Montreal, Quebec). 10,000 pfu were screened from the Brisson library. The 131 bp DNA was random primed using the NEBlot Kit (NEB) and [α-^{32}P]dATP (NEN, 3000 C_i/mmole) to a specific activity of ~10^9 dpm/μg. Hybridization and washing were done as described by Maniatis *et al.* (25). A single insert was isolated, and the Bluescript plasmid was excised using the ExAssist/SOLR *in vivo* excision protocol (Stratagene). The cDNA was sequenced on both strands with regularly spaced internal sense and antisense primers.

Nucleic acid and amino acid sequence alignments. The apyrase DNA sequence and the deduced amino acid sequence were checked for homology to known sequences using the NCBI Blast E-mail server. The *BlastP* program was used for protein sequence and *BlastN* was used for DNA sequence (27). All sequence comparisons and analyses were done using the Genetics Computer Group (GCG) software.

Northern and Southern blot analysis. 40 μg total RNA isolated from potato tubers (26) was run on a 1% denaturing agarose gel and transferred to GeneScreen Nylon Membranes (NEN) using capillary action as described by Maniatis *et al.* (25). Prehybridization and hybridization were performed in 50% formamide and washing was performed in SSC and SDS as described by Maniatis *et al.* (25).

Genomic DNA from potato leaves (var. Red dale) and *Arabidopsis* leaves (generously supplied by Dr. Robert Pruitt, Harvard University, Cambridge, MA) was isolated using the GREEN-GENE Plant DNA Isolation Kit (Clontech). 25 μg of genomic DNA from potatoes was digested with 150 Units of EcoRI and HindIII in separate reactions, and 25 μg of genomic DNA from *Arabidopsis* was digested with 150 Units of EcoRI. Prehybridization and hybridization were carried out as described above. For the *Arabidopsis* Southern, 30% formamide was used.

RESULTS

Purification of apyrase. The potato tuber apyrase was isolated as described by Kettlun *et al.* (18) with an additional sucrose gradient centrifugation step (Table 1). Starting with 1 kg of peeled red skin potatoes, a four step protocol was used to enrich the specific activity of the apyrase 10,000-fold from ~1 μmol P_i/mg/min in the crude extract to ~10,000 μmol P_i/mg/min. At this stage of the purification, SDS-PAGE showed a main band at ~50 kDa and a minor band at ~40 kDa (Fig. 1). The molecular mass of the native and denatured protein has been determined to be ~45–50 kDa

TABLE 1
Potato Apyrase Purification Protocol

Purification step	Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/mg/min)	Purification (-fold)	Yield (%)
Crude extract	13,000	13,500	1	NA	100
$(NH_4)_2SO_4$ fractionation	25	2,000	80	80	15
Gel filtration column	0.52	1,000	2,000	25	7.5
Cibracon blue column	0.059	350	6,000	3	2.5
Sucrose gradient	0.0015	15	10,000	1.7	0.1

using irradiation inactivation rates (28), gel filtration (18), sedimentation velocities (29), and SDS polyacrylamide gel electrophoresis (19). Assuming a molecular mass of 50 kDa and using the reported K_M for ATP of 10^{-4} M in the presence of Ca^{2+} (18), we estimate a turnover number of $\sim 10^4$ s^{-1} and a $k_{cat}K_M$ of $\sim 10^8$ $M^{-1}s^{-1}$.

The 50 kDa band was digested with trypsin and five peptides were sequenced. No amino terminal sequence could be obtained. Nucleotide sequences corresponding to the two longest peptides (Fig. 2, peptides #1 and #2) were amplified by PCR. Subsequent attempts to amplify the region between these two DNA sequences resulted in a 131 bp product and the discovery that the peptides are adjacent to each other. Tryptic digestion of the 40 kDa band gave an almost identical HPLC peptide map to that of the 50 kDa band (data not shown) indicating that both represent the same protein with the 40 kDa band most likely a degradation product.

cDNA library screening. The 131 bp cDNA sequence corresponding to most of the region coding for peptides #1 and #2 was used to probe a potato tuber cDNA library generously given to us by Dr. Normand Brisson (University of Montreal, Quebec). A single clone consisting of 1530 bp was isolated. The first ATG occurs 45 bp in from the 5′ end and is assumed to be the translation initiation site based on two criteria: the upstream sequence is not too divergent from the Kozak initiation site consensus sequence (30), and the observed molecular mass of $\sim$50 kDa and the

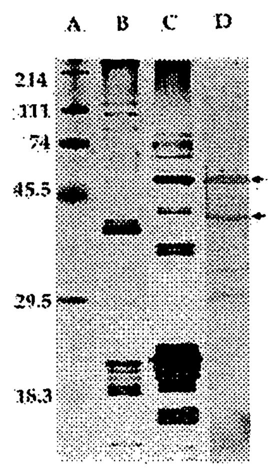

FIG. 1. SDS-PAGE of the potato apyrase purification fractions. Various steps of the purification protocol were analyzed on a 12.5% Laemmli gel (43). The protein bands were silver stained as described by Ansorge (44). (A) Prestained molecular weight markers with the apparent molecular masses indicated on the left in kilodaltons. (B) Approximately 5 μg of total protein from the crude extract. (C) Approximately 5 μg of total protein after the third ammonium sulfate fractionation step. (D) Approximately 10% of the sucrose density gradient fraction exhibiting the highest specific activity. The main 50 kDa and minor 40 kDa bands are indicated by arrows.

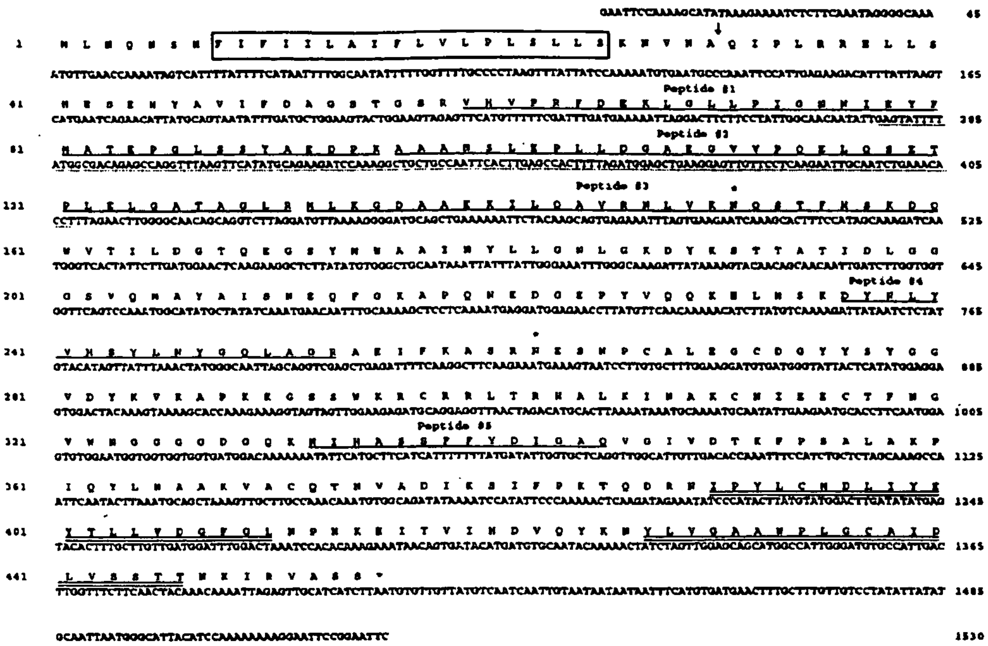

FIG. 2. Nucleotide and deduced amino acid sequence of the potato apyrase cDNA, RROP1. The peptides isolated by microsequencing are single underlined. The boxed amino acids represent the putative signal sequence with the predicted cleavage site indicated by the arrow (32). The two regions of hydrophobicity at the carboxy terminus are double underlined. Asterisks locate the potential Asn-linked glycosylation sites. The numbers on the left represent amino acids and the numbers on the right represent nucleotides. The nucleotide sequence single dot underlined (277–407) represents the region used as the probe in the screening of the cDNA library and all Northern and Southern blot analyses.

calculated molecular mass of 49.9 kDa from the deduced polypeptide sequence of 454 amino acids are in good agreement (Fig. 2). The deduced amino acid sequence contains all five of the peptides sequenced (Fig. 2). There is no consensus polyadenylation signal.

Hydropathy analysis using the Kyte-Doolittle algorithm (31) predicts three hydrophobic stretches in the protein. The first one at the amino terminus (residues (8–25) appears to represent a signal peptide with a predicted cleavage site after Ala-30 (32). The other two in the carboxy terminus are stretches of 21 amino acids (residues 390–410) and 20 amino acids (residues 427–446). Both are sufficiently long to traverse a membrane but whether they represent actual transmembrane domains has not been determined. There are two potential Asn-linked glycosylation sites at Asn-151 and Asn-262. Treatment of the purified apyrase with either N-glycanase or neuraminidase had no effect on the mobility of the protein in an SDS polyacrylamide gel indicating the protein is not heavily glycosylated (data not shown).

Sequence alignment. Using the NCBI *BlastP* program (27), seven similar protein sequences were identified: the two isoforms of an NTPase, NTP1 and NTP3, from a protozoan parasite (*Toxoplasma gondii*) (5), a nucleoside triphosphatase from garden pea (*Pisum sativum*) (33), a yeast golgi guanosine diphosphatase (*Saccharomyces cerevisiae*) (34), human CD39 (35), a hypothetical 71.9 kDa yeast protein (36), and a hypothetical 61.3 kDa protein from *Caenorhabditis elegans* (37). The potato apyrase showed 49, 33, 25, 23, 23, and 22% amino acid identity and 67, 52, 48, 52, 50, and 48% similarity with the garden pea NTPase, yeast GDPase, human CD39, yeast hypothetical protein, both *Toxoplasma* NTPases, and the *C. elegans* hypothetical protein, respectively. Analysis of all the sequences retrieved revealed four well conserved regions which we term ACR (apyrase

conserved regions) 1–4 (Fig. 3). None of the putative conserved sites show any striking similarity to the consensus Walker ATP binding motifs (38–40), but conserved region #1 and #4 are similar to the actin-hsp 70-hexokinase β-and γ-phosphate binding motifs, respectively (5,41).

The NCBI *BlastN* program (27) identified two sequences similar to the potato apyrase at the nucleic acid level: the garden pea NTPase (33) and a partial rice cDNA sequence (42).

Northern and Southern blot analysis. Total RNA isolated from potato tubers was probed with the 131 bp cDNA fragment (nucleotides 277–407). One band at ~1500–1600 bp was seen indicating a single RNA transcript of similar size to the cDNA clone (Fig. 4A). 25 μg of genomic DNA isolated from potato leaves was digested with EcoRI and HindIII. Southern blots of the digests showed multiple bands (Fig. 4B) indicating the potato apyrase is multigenic based on the observations that the restriction enzymes do not cut within the coding region and the 131 bp cDNA used as probe does not contain introns within the fragment at the level of the genomic DNA (data not shown). Genomic DNA from *Arabidopsis* was also examined under lower stringency hybridization and wash conditions; two bands were seen using the same probe as for the potato Southern analysis (Fig. 4C).

DISCUSSION

This manuscript describes the purification of a potato apyrase and the cloning of a cDNA encoding the potato enzyme. The identification of the cDNA as the apyrase gene is based on several criteria. First, the five peptides isolated from the purified apyrase are all present in the deduced polypeptide sequence. Second, the polypeptide is similar in sequence to known apyrases and related enzymes. Third, each of the four putative apyrase conserved regions share nearly identical sequence and spatial conservation with the related proteins (Fig. 3).

Although no functional studies on the ACR regions are available, the most reasonable hypothesis is that the conserved sites are involved in the catalytic activity of the apyrase. First, all of the similar proteins with known activities hydrolyze phosphoanhydride bonds of nucleoside tri- and di- phosphates (apyrases from potato, garden pea, and *T. gondii*) or nucleoside diphosphates (yeast GDPase). Second, ACR1 and ACR4 are similar to the actin-hsp 70-hexokinase β- and γ-phosphate binding motifs (5,41), indicating a possible role in nucleotide binding.

The mosquito apyrase which was recently cloned shows little similarity to the potato apyrase at the amino acid level, including the regions corresponding to the ACR sequences (Fig. 3). Champagne *et al.* (7) indicated that the deduced amino acid sequence of the salivary apyrase cDNA was similar to that of the 5'-nucleotidase protein family instead. Although the purified mosquito apyrase exhibits catalytic activity comparable to that of the other apyrases that have been cloned, it appears that this insect enzyme either evolved from a different ancestral progenitor than that for the proteins containing the ACR sites or that the cloned mosquito cDNA is a 5'-nucleotidase rather than an apyrase.

Interestingly, a yeast hypothetical 71.9 kDa protein sequenced from chromosome V, a *C. elegans* hypothetical 61.3 kDa protein sequenced from chromosome III, and a human lymphoid cell activation antigen, CD39, contain all four ACR regions and show high similarity with the apyrases at the amino acid level (Fig. 3). CD39 is primarily expressed on activated lymphoid cells and has been shown to mediate homotypic adhesion of B cells in the presence of anti-CD39 monoclonal antibodies (36). Currently, no enzymatic activity has been attributed to CD39, yet the possibility that this protein may also be responsible for the ecto-ATPase activity in human lymphoid cells (1) is intriguing.

Potato apyrase exists in both soluble and insoluble states that differ in their catalytic properties, isoelectric points, and slightly in molecular weights (15–19). We were only able to isolate a single cDNA from a potato tuber library, but Southern analysis on potato genomic DNA indicated the existence of multiple genes (Fig. 4B). Either there is only one active gene that can produce both isoforms or we have not isolated all the apyrase cDNA sequences.

A

Potato Apyrase	44	E H Y A V I F D A G S T G S R V H V F R F D	65
Garden Pea NTPase	42	S S Y A V V F D A G S T G S R I H V Y H F N	63
S. cerevisiae GDPase	91	H K Y V I M I D A G S T G S R V H I Y K F D	112
T. gondii NTP1	63	L Q A L V V I D A G S S S T R T N V F . L A	83
T. gondii NTP3	63	L Q A L V V I D A G S S S T R T N V F . L A	83
Yeast 71.9 kDa	8	D R F G I V I D A G S S G S R I H V F K W Q	29
C. elegans 61.3 kDa	42	R S Y G V I C D A G S T G T R L F V Y N W I	63
Human CD39	47	V K Y G I V L D A G S S H T S L Y I Y K W P	68
Murine CD39	47	V K Y G I V L D A G S S H T N L Y I Y K W P	68
Mosquito Apyrase	32	K D V S K L F P L T L I H I N D L H A R F E	53

B

Potato Apyrase	120	. T P L E L G A T A G L R N L K G D . .	136
Garden Pea NTPase	118	. T P V R L G A T A G L R L L N G D . .	134
S. cerevisiae GDPase	165	. T P V A V K A T A G L R L L G D A . .	181
T. gondii NTP1	180	G I P V M L C S T A G V R D F H E . . .	196
T. gondii NTP3	180	G I P V M L C S T A G V R D F H E . . .	196
Yeast 71.9 kDa	100	. C P V F I Q A T A G M R L L P Q D I .	117
C. elegans 61.3 kDa	124	. T P V F I F A T A G M R L I P D E Y V	142
Human CD39	124	. T P V Y L G A T A G M R L L R M E S E	142
Murine CD39	124	. T P V Y L G A T A G M R L L R M E S E	142
Mosquito Apyrase	150	E G I P T I V A N L V M N N D P D L K S	169

C

Potato Apyrase	165	. L D G T Q E G S Y M W A A I N Y L L G N L G K	186
Garden Pea NTPase	163	. I D G T Q E G S Y L W V T V N Y A L G N L G K	184
S. cerevisiae GDPase	211	. M G G D E E G V F A W I T T N Y L L G N I G A	232
T. gondii NTP1	230	P I T G A E E G L F A F I T L N H L S R R L G E	253
T. gondii NTP3	230	P I T G A E E G L F A F I T L N H L S R R L G E	253
Yeast 71.9 kDa	147	. I D G E T E G L Y G W L G L N Y L Y G H F N D	169
C. elegans 61.3 kDa	174	. I E G K W E G I Y S W I A V N Y A L G K F N K	196
Human CD39	169	. I T G Q E E G A Y G W I T I N Y L L G K F S Q	183
Murine CD39	169	. I T G Q E E G A Y G W I T I N Y L L G R F T Q	183
Mosquito Apyrase	205	T L S N A V E A V . R R E A A A L K K D K I D I	227

D

Potato Apyrase	192	. T T A T I D . . L G G G S V Q M A Y A I S N E	212
Garden Pea NTPase	190	. T V G V I D . . L G G G S V Q M A Y A V S K K	210
S. cerevisiae GDPase	239	P T A A V F D . . L G G G S T Q I V F . . . E P	157
T. gondii NTP1	271	D L A G V V E . . V G G A S A Q I V F P L Q E G	192
T. gondii NTP3	271	D L A G V V E . . V G G A S A Q I V F P L Q E G	192
Yeast 71.9 kDa	178	F T F G F M D . . M G G A S T Q I A F A P H D S	199
C. elegans 61.3 kDa	213	K T V G M I D . . M G G A S A Q I A F E L P D T	234
Human CD39	207	E T F G A L D . . L G G A S T Q V T F V P Q N Q	228
Murine CD39	207	E T F G A L D . . L G G A S T Q I T F V P Q N S	228
Mosquito Apyrase	245	E A G D D I D V I V G A H S H S F L Y S P D S K	268

FIG. 3. Sequence analysis of the putative apyrase conserved regions (ACRs) of the potato apyrase and related proteins. The amino acid sequences from potato apyrase, garden pea nucleoside triphosphatase, *Saccharomyces cerevisiae* golgi guanosine diphosphatase, *Toxoplasma gondii* nucleoside triphosphate hydrolase isoforms (NTP1 and NTP3), a yeast hypothetical 71.9 kDa protein, a *Caenorhabditis elegans* hypothetical 61.3 kDa protein, human CD39, murine CD39 (35), and mosquito salivary apyrase were aligned using the GCG *Pileup* program. The boxed areas represent the core regions of high homology. (A) Putative apyrase conserved region #1 (ACR1). (B) ACR2. (C) ACR3. (D) ACR4.

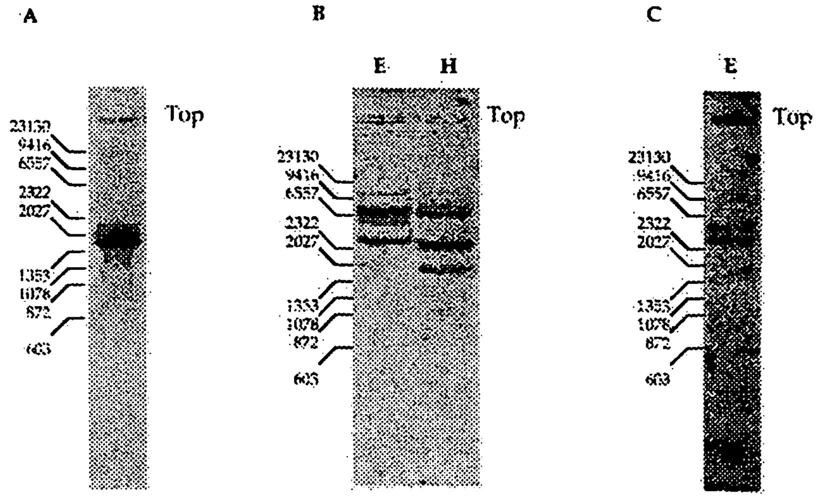

FIG. 4. Northern and Southern blot analysis in potatoes and *Arabidopsis*. All blots were probed with the 131 bp PCR product, nucleotides (277–407). The top of each gel is labeled. The molecular markers are indicated on the left in base pairs. (A) Northern analysis of 40 μg total RNA from potato tubers. (B) Southern analysis of 25 μg potato genomic DNA digested with EcoRI(E) and HindIII(H). (C) Southern analysis of 25 μg *Arabidopsis* genomic DNA digested with EcoRI(E).

In *T. gondii*, the mature forms of the NTP1 and NTP3 gene products are soluble proteins that are able to associate with membranes. The isoforms are 97% identical at the amino acid level and are predicted to undergo cleavage of their putative signal sequences based on the reduced molecular mass of the *in vitro* translated NTP1 polypeptide in the presence of microsomes (5). Immunofluorescence studies showed that both NTPase were located within dense granules of the protozoan parasite and upon host cell infection were secreted into the parasitophorous vacuole where a fraction of both enzymes remained soluble and a fraction associated with the parasitophorous vacuolar membrane (PVM) (5). It has been shown that one of the membrane associated apyrases from potato can be fully solubilized in 2 M NaCl indicating a peripheral attachment to the membrane as opposed to an integral one (19).

We are currently attempting to express an active potato apyrase in COS-7 cells and are planning to isolate related genes in animal cells by cloning through homology. The possibility of an apyrase homolog in *Arabidopsis* and *C. elegans* now opens up avenues for genetic manipulations to study the physiology of the apyrases.

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